A PRELIMINARY MICROSATELLITE LINKAGE MAP OF THE RED DRUM (*SCIAENOPS OCELLATUS)*

A Senior Scholars Thesis

by

CHRISTOPHER M. HOLLENBECK

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Biology

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Approved by:

Research Advisor: John R. Gold
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

A Preliminary Microsatellite Linkage Map of the Red Drum (*Sciaenops ocellatus*).
(April 2009)

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A genetic linkage map was generated for the red drum (*Sciaenops ocellatus*), a marine fish species of considerable economic importance in the southeastern United States. Two single-pair mating families of 104 progeny were genotyped at 60 nuclear-encoded microsatellites and analysis of the data enabled identification of 13 linkage groups. The linear arrangement of the microsatellites within each linkage group was determined and map distances between adjacent markers were estimated. Significant family- and sex-specific differences in recombination rates between adjacent loci were found. This project represents the beginning of a microsatellite-based linkage map for red drum.

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NOMENCLATURE

Gulf	Gulf of Mexico
LG	Linkage Group
TPWD	Texas Parks and Wildlife Department

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CHAPTER I

INTRODUCTION

Overview of red drum

The red drum (*Sciaenops ocellatus*) is a marine fish species of the family Sciaenidae that populates bays and estuaries in the northern Gulf of Mexico (hereafter Gulf) and along the Atlantic coast of the United States (Pattillo *et al.* 1997). Red drum have traditionally been a popular saltwater game fish in the southeastern United States, especially in states bordering the Gulf (Van Voorhees *et al.* 1992). In Texas, conflict between recreational and commercial interests for access to the fishery and reports of decreasing red drum abundance influenced the Texas legislature to ban the sale of commercially caught red drum (Christian 1986). In response to dwindling abundance occurring over the past few decades, many Gulf and Atlantic coast states established culture and stock-enhancement programs that sought to maintain an abundant supply of red drum in coastal regions. Specifically, the Texas Parks and Wildlife Department (TPWD) began in the 1980s a stock-enhancement program that now supports the red drum fishery in Texas waters through annual releases of over 30 million hatchery-raised fingerlings into eight different Texas bays and estuaries (McEachron *et al*. 1995). Red drum have also become a popular species for private aquaculture. In addition to multiple operations in the southern United States, private red drum aquaculture facilities can be found as well in Taiwan, Israel, and mainland China (Lutz 1999).

This thesis follows the style of Genetics.

Project overview

As of today, medium to dense genetic linkage maps have been developed for many economically important aquaculture species, including rainbow trout (Sakamoto and Danzmann 2000), channel catfish (Waldbieser *et al*. 2001), the Pacific oyster (Hubert and Hedgecock 2004), and tilapia (Kocher *et al*. 1998; Lee *et al*. 2005). These maps have been generated using various types of genetic markers, including amplified fragment-length polymorphisms (AFLPs), randomly amplified polymorphic DNA markers (RAPDs), simple-sequence repeats (SSRs, including microsatellites), and most recently, single nucleotide polymorphisms (SNPs). Microsatellites are relatively short, highly polymorphic, nuclear-encoded genetic markers that consist of abundant, short stretches of di-, tri, or tetranucleotide repeats embedded within unique flanking regions (Weber and May 1989; Wright and Bentzen 1994). Once suitable polymerase-chain-reaction (PCR) primers are designed for a given microsatellite, allelic variation at that microsatellite can be assessed using PCR amplification, followed by gel electrophoresis of the amplification products. As a PCR-based marker, microsatellites are easily amplified, highly reproducible, and co-dominantly inherited (Weber 1990; Wright and Bentzen 1994). This makes them ideal for the construction of genetic linkage maps (Liu and Cordes 2004).

This paper details the generation of a red drum genetic linkage map that contains 60 microsatellites. Details (e.g., primer sequences, number of alleles) regarding each microsatellite may be found in Saillant *et al.* (2004) and Karlsson *et al.* (2008). The map

consists of the 31 microsatellites previously mapped by Karlsson *et al.* (2007) and 29 'new' microsatellites that have not been previously assigned to red drum linkage groups. In addition, comparisons of rates of genetic recombination between linked microsatellites were made between two mapping families and between sexes. The map will provide the framework for future linkage studies that incorporate many additional markers, with the ultimate goal of developing a moderately dense linkage map that covers each of the 24 linkage groups known from cytological studies in the species (Gold *et al*. 1988).

CHAPTER II

METHODOLOGY

Data collection

Collection of data was completed by determining genotypes at each of 60 nuclear-encoded microsatellites among parents and progeny in each of two mapping families (Family A and Family B). Each family was generated from P_1 crosses between of a single sire and a single dam, and a total of 104 F_1 progeny from each family were assayed. Crosses and rearing were performed at the CCA/CPL Marine Development Center of the Texas Parks and Wildlife Department in Flour Bluff, Texas. Genomic DNA from each fish was extracted from a small sample of fin or muscle tissue preserved in ethanol, using a PCI or chelex resin extraction protocol. PCR amplification of microsatellites utilizing fluorescent and tail-labeled primers was performed with two slightly different protocols.

PCR amplification of microsatellite loci using fluorescently-labeled primers

PCR amplifications for eight of the microsatellites (Table 1) utilized PCR primer pairs in which either the forward or reverse primer was end-labeled with the [^{32}P]γ-dATP fluorescent dye HEX or FAM (Invitrogen). For each microsatellite, the reaction consisted of a 10 μL volume containing 5 pmol of each primer (forward and reverse), 2.0 mM of $MgCl_2$, 2.0 mM of each dinucleoside triphosphate, 2 μL of 5x reaction buffer, 1 μL of DNA, and 0.5 units of Go*Taq* DNA polymerase. Reactions were run using a PTC-200

TABLE 1
29 Microsatellites Used in the Construction of a Linkage Map for Red Drum (*Sciaenops ocellatus*)

Fluorescent Primers		
Microsatellite	**Repeat Sequence of Original Isolate**	**Number of Alleles**
Soc 49	$(CA)_{24}$	-
Soc 409	$(TG)_{11}$	7
Soc 418	$(TG)_{24}$	22
Soc 421	$(TG)_{34}$	10
Soc 429	$(TG)_{12}$	4
Soc 431	$(TG)_{29}$	8
Soc 434	$(CA)_{23}$	7
Soc 439	$(TG)_{17}$	4
Tail-Labeled Primers		
Microsatellite	**Repeat Sequence of Original Isolate**	**Number of Alleles**
Soc 635	$(CA)_{23}$	10
Soc 636	$(CA)_{35}$	25
Soc 637	$(CA)_{31}$	20
Soc 638	$(GA)_{14}$	5
Soc 639	$(CA)_{13}$	7
Soc 645	$(CA)_{20}$	10
Soc 646	$(CA)_{16}$	4
Soc 648	$(CA)_5(N)_2(CA)_{22}$	14
Soc 650	$(CA)_{22}$	14
Soc 651	$(CA)_{23}$	20
Soc 653	$(CA)_{26}$	17
Soc 656	$(CA)_{24}$	23
Soc 659	$(CA)_{17}$	10
Soc 662	$(CA)_{20}(N)_2(CA)_6(N)_2(CA)_3$	13
Soc 664	$(CA)_{23}$	15
Soc 725	$(CA)_{15}$	9
Soc 726	$(CA)_{15}(N)_2(CA)_4(N)_2(CA)_{11}$	16
Soc 729	$(CA)_9(N)_2(CA)_4$	13
Soc 735	$(CA)_{31}$	23
Soc 738	$(CA)_{28}$	21
Soc 739	$(CA)_3(N)_2(CA)_3(N)_2(CA)_{10}$	16

Data for fluorescent primer microsatellites are summarized in Saillant *et al*. (2004).
Data for tail primer microsatellites are summarized in Karlsson *et al*. (2008).

(MJ Research) or MyCycler (Bio-Rad) thermocycler, with an initial denaturation for five minutes at 95C, followed by 30 cycles consisting of 45 seconds of denaturation at 95C, 45 seconds at the primer-specific annealing temperature, and 60 seconds at 72C, and an extension period of 10 minutes at 72C.

PCR amplification of microsatellite loci using tail-labeled primers

PCR amplifications of the remaining 21 microsatellites (Table 1) employed tail-labeled primers. This approach for PCR amplification of microsatellites is a cost-effective alternative to the traditional method that involves direct labeling of either forward or reverse primer. In this method, the forward primer is 5'-tailed with an arbitrary primer sequence, in this case taken from the microsatellite primer sequence CATR8 used in the PCR amplification of genomic DNA from the Madagascar periwinkle, *Catharanthus roseus* (Shokeen *et al*. 2007). In addition to the tail-labeled 5' (forward) and 3' (reverse) primers, a third primer consisting of a ^{32}P-labeled tail sequence is added to the reaction. This primer can be used with any tail-labeled forward primer (and its complementary unlabeled reverse primer) to amplify and fluorescently label a locus (Boutin-Ganache *et al*. 2001). Because a primer for each individual locus does not need to be fluorescently-labeled, this is a very cost-effective method of genotyping for projects involving many markers. For the 21 microsatellites amplified using tail-labeled primers (Karlsson *et al*. 2008), each reaction consisted of a 10 μL volume containing 5.0 pmol each of the reverse primer and tail primer labeled with FAM or NED fluorescent dye), 0.5 pmol of the tail-labeled forward primer, 2.0 mM of $MgCl_2$, 2.0 mM of each dinucleoside

triphosphate, 2 μL of 5x reaction buffer, 1 μL of DNA, and 0.5 units of *GoTaq* DNA polymerase. The PCR protocol consisted of an annealing temperature step-down in which each reaction was run using a three-step decrease in annealing temperature. The protocol consisted of ten cycles with an annealing temperature of 58C, ten cycles at 55C, and ten cycles at 52C. The periods of denaturation and extension were the same as in the previously mentioned protocol. Following amplification, electrophoresis was performed using an automated ABI-377 sequencer. Alleles were scored using the software GENOTYPER version 2.5.

PCR amplification of 31 previously-mapped Loci

All individuals in both mapping families were genotyped at the 31 microsatellites that were previously mapped by Karlsson *et al*. (2007). Amplification of these loci was performed using a series of six multiplexed PCR reactions (described in Renshaw *et al.* 2006).

Data analysis

Following the genotyping of each individual in both mapping families, the software package LINKMFEX (available at http://www.uoguelph.ca/~rdanzman/software/LINKMFEX) and the suite of programs included therein were used to analyze the data. First, a logarithm-of-the-odds (LOD) score was assigned to each pair of markers being analyzed. An LOD score is a statistic that represents the likelihood that a pair of loci are linked, based on sample size and the

estimated rate of recombination between the two loci (Liu 2007). Microsatellite pairs with a calculated LOD score of 3.0 or higher were inferred to be linked. With this information for each pair of microsatellites, it was possible to define individual linkage groups and to arrange microsatellites in the most likely linear configuration along the length of each identified linkage group. A map distance, measured in θ units and equal to the incidence of recombination in the interval between two linked microsatellites, was then estimated. From this information a linkage map was constructed and displayed graphically with the program MAPCHART (Voorrips 2002). This complete process was carried out for each mapping parent (female and male) in both mapping families (A and B). Following this, the sex-specific maps were merged to provide both a female- and male-specific map with map distances between adjacent microsatellites averaged across the two families.

Comparisons of recombination rates between families and sexes were performed using the program RECOMDIF, a program included within the LINKMFEX package. RECOMDIF was used to compare estimated recombination rates between adjacent marker pairs in the family- and sex-specific maps. The program presented a ratio of recombination for each adjacent pair of markers shared between the two maps under comparison and tested the significance of the difference using a contingency G-test (Sokal and Rohlf 1981). As per the specifications of the program, two recombination estimates were considered to differ significantly if the G-test value exceeded 3.84. If the sample size was smaller than five, a G-test value incorporating Williams' correction for small sample sizes was used.

CHAPTER III

RESULTS

Linkage map

The male and female parents of Family A were heterozygous for the same alleles at *Soc*60, *Soc*410, and *Soc*433. The male and female parents of Family B either possessed the same heterozygous genotype or were monomorphic at *Soc*140, *Soc*407, *Soc*415, *Soc*433, *Soc*444, *Soc*445, and *Soc*659. Consequently, the parental phases of the alleles could not be identified and the microsatellites were omitted from further analysis in the respective families. The genetic linkage map (Figure 1) shows linkage relationships (θ distances) for comparative sex-specific linkage groups, each averaged across the two families. A total of 13 linkage groups (LG) were identified based on LOD score of 3.0 or greater. LGs 1-9 were numbered based on the previous linkage study (Karlsson *et al*. 2007) in order to maintain a degree of continuity for future linkage studies in red drum; LGs 10-13 were identified based on the results of this study. The LOD scores for several microsatellites indicated independent assortment relative to all other markers in one or both families. Seven microsatellites (*Soc*85, *Soc*99, *Soc*423, *Soc*635, *Soc*637, *Soc*651, and *Soc*664) displayed no linkage relationship with any of the remaining 53 microsatellites in either the male- or female-specific maps. Four microsatellites (*Soc*432, *Soc*638, *Soc*645, and *Soc*738) displayed no linkage relationships in the female-specific map; while 11 microsatellites (*Soc*19, *Soc*201, *Soc*404, *Soc*419,

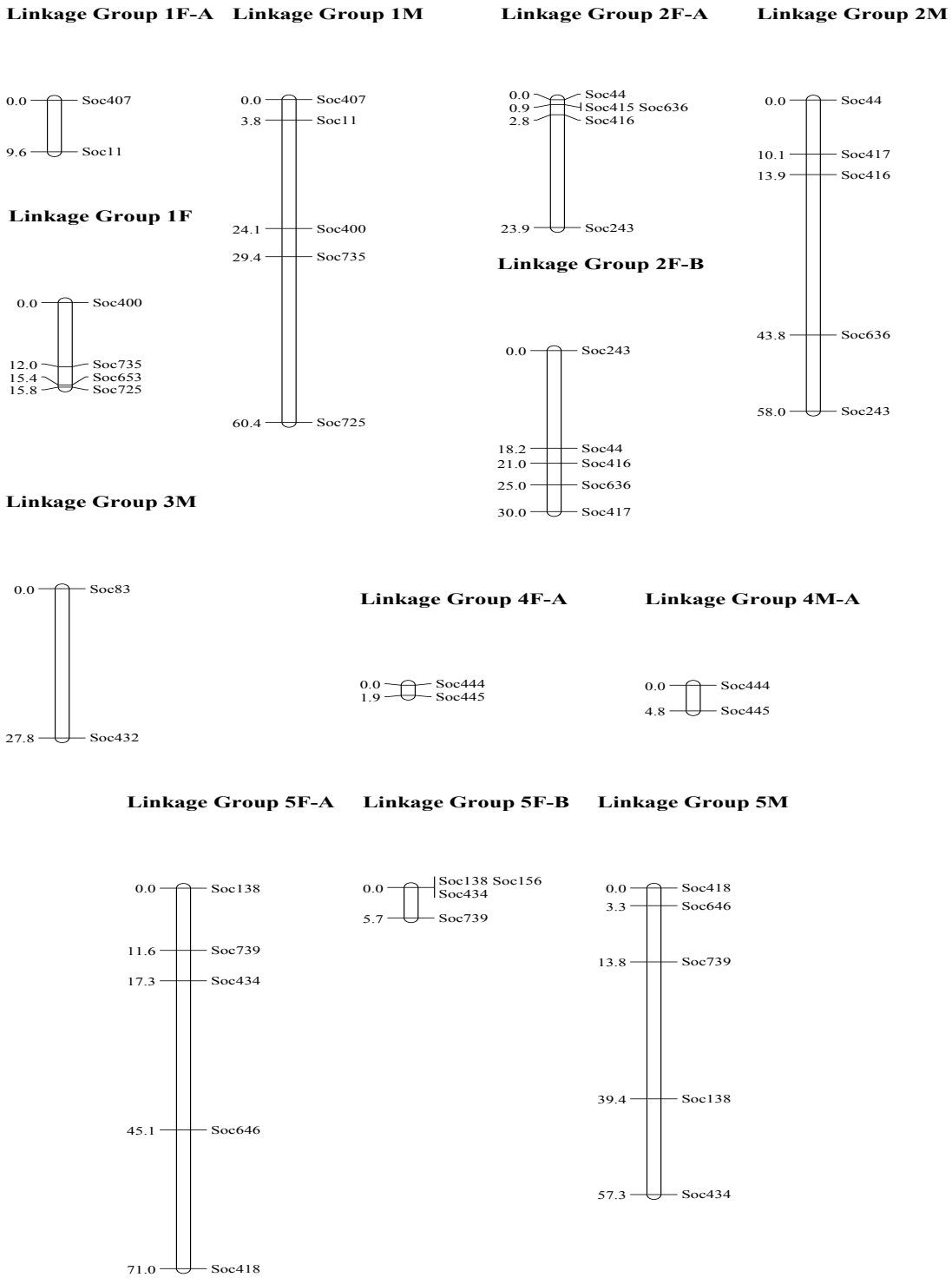

FIGURE 1- A genetic linkage map of *Sciaenops ocellatus*. Linkage groups denoted with an "F" represent female-specific linkage groups, and groups denoted with an "M" represent male-specific linkage groups. An "A" or "B" denotation marks groups that were not averaged across families, but rather were derived solely from family A or B, respectively.

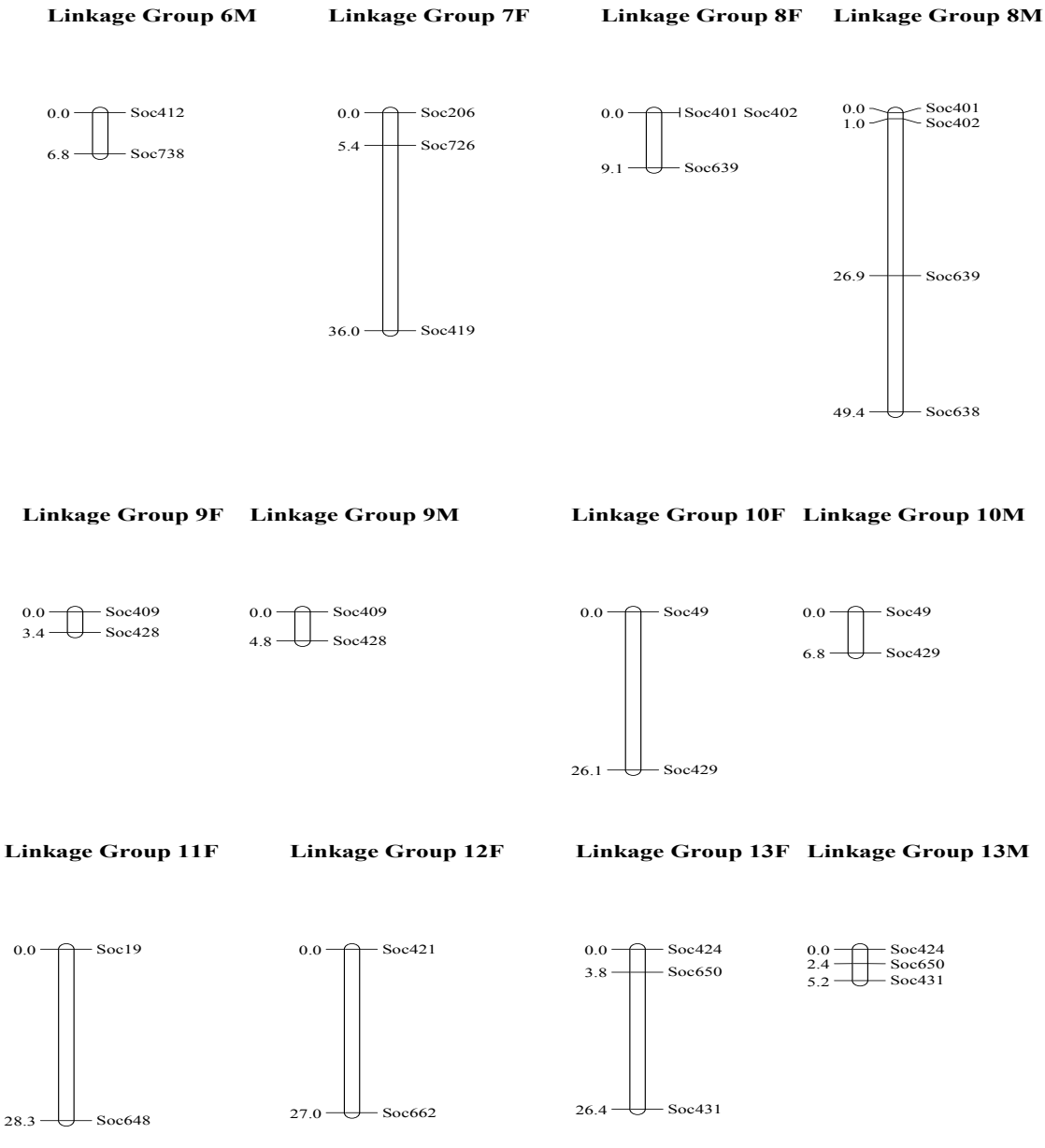

FIGURE 1- *Continued*

*Soc*421, *Soc*439, *Soc*648, *Soc*656, *Soc*662, *Soc*726, and *Soc*729) displayed no linkage relationship in the male-specific map.

LGs 7, 11, and 12 were identified only in the female map, while LGs 3 and 6 were identified only in the male map. For various reasons, map distances in several LGs could not be averaged across the two families. As an example, based on comparison to the male map, LG1F-A is most likely associated with LG1F; however, merging of these groups was not possible as no linkage relationship was established between *Soc*11 and *Soc*400. In two instances (LG2F and LG5F), female-specific linkage groups were unable to be merged due to conflicting marker order. In addition, LG 4 was only identified in Family A as a result of uninformative parental genotypes at *Soc*444 and *Soc*445 in Family B.

Differential recombination rates

Family-specific differences in recombination

Estimated recombination ratios (Family A:Family B) for adjacent markers in both male- and female-specific maps are given in Table 2. Values shown in bold represent ratios with significantly different recombination rates between families based on contingency G-tests. A zero value in the table indicates that the recombination rate between the two markers in Family A was zero. A zero value denoted with a "*" indicates a case where pair-wise recombination was zero in Family B. While these particular values are actually undefined (a ratio cannot have zero for a denominator) they are reported as zero "*" for

clarification purposes. “N/a” is listed for microsatellite pairs that were unavailable for comparison as a result of uninformative parental genotypes, etc.

Sex-specific differences in recombination

Estimates of recombination ratios between sexes (♀:♂) for adjacent pairs of markers in Family A and Family B are reported in Table 3. The notation for this table is consistent with that of Table 2, with the exception that a zero value in Table 3 represents a case where there was zero recombination between the microsatellite pair in the female parent. Similarly, a zero value denoted with a “*” indicates zero recombination between a pair of markers for the male parent.

TABLE 2
Recombination Ratios Between Families (A:B)

Marker Pair	♀♀	♂♂
Soc 11-Soc 400	n/a	0.919
Soc 400-Soc 735	1.286	**10.778**
Soc 735-Soc 653	0.396	n/a
Soc 653-Soc 725	0.000*	n/a
Soc 44-Soc 417	n/a	0.748
Soc 44-Soc 636	0.300	n/a
Soc 417-Soc 416	n/a	**7.444**
Soc 416-Soc 243	1.099	n/a
Soc 636-Soc 243	n/a	0.893
Soc 636-Soc 416	0.475	0.929
Soc 83-Soc 432	n/a	1.000
Soc 138-Soc 739	2.035	1.048
Soc 138-Soc 434	**0.000***	1.190
Soc 739-Soc 434	1.000	n/a
Soc 646-Soc 739	n/a	2.149
Soc 418-Soc 646	n/a	6.333
Soc 412-Soc 738	n/a	1.812
Soc 206-Soc 726	2.821	n/a
Soc 401-Soc 402	n/a	0.000*
Soc 401-Soc 639	n/a	1.158
Soc 402-Soc 639	1.716	1.158
Soc 638-Soc 639	n/a	0.622
Soc 409-Soc 428	**0.000***	**9.667**
Soc 49-Soc 429	0.816	1.368
Soc 19-Soc 648	0.965	n/a
Soc 421-Soc 662	0.759	n/a
Soc 424-Soc 650	3.000	**0.000***
Soc 424-Soc431	n/a	2.714
Soc 650-Soc 431	1.349	1.000
Average	1.203	**1.185**

TABLE 3
Recombination Ratios Between Sexes (♀:♂)

Marker Pair	Family A	Family B
Soc 11-Soc 407	2.526	n/a
Soc 400-Soc 735	1.392	**11.667**
Soc 735-Soc 725	**0.094**	n/a
Soc 44-Soc 416	n/a	**0.224**
Soc 44-Soc 243	n/a	**0.421**
Soc 44-Soc 636	**0.028**	n/a
Soc 636-Soc 417	n/a	**0.167**
Soc 636-Soc 416	**0.066**	**0.129**
Soc 416-Soc 243	**0.499**	n/a
Soc 444-Soc 445	0.396	n/a
Soc 138-Soc 739	**0.443**	**0.228**
Soc 138-Soc 434	n/a	**0.000**
Soc 739-Soc 434	**0.156**	**0.145**
Soc 434-Soc 646	**0.590**	n/a
Soc 646-Soc 418	**4.544**	n/a
Soc 401-Soc 402	0.000	n/a
Soc 401-Soc 639	**0.414**	n/a
Soc 402-Soc 639	**0.414**	**0.279**
Soc 409-Soc 428	0.770	0.000
Soc 49-Soc 429	**3.013**	**5.053**
Soc 424-Soc 431	n/a	**6.857**
Soc 424-Soc 650	1.188	0.000*
Soc 650- Soc 431	**9.250**	**6.857**
Average	**0.593**	**0.550**

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Linkage map and differences in rates of recombination

A total of thirteen linkage groups were identified in this study, including four that were not identified by Karlsson *et al*. (2007). The red drum genome has a haploid set of 24 chromosomes (Gold *et al.* 1988), meaning that additional markers will be required to resolve the entire complement of chromosomes. It also is possible that some of identified linkage groups will be merged as more markers are added. Generally, it is recommended (Liu 2007) that a Kosambi or Haldane function be applied to θ estimates of map distances to correct for multiple crossovers between markers. This is because as more linkage relationships are established and the distance between terminal loci within a linkage group increases, the possibility of multiple crossovers between distant loci increases as well. This causes an overall underrepresentation of recombination, because double crossovers reverse the linkage phase of recombinant genotypes (Liu 2007). In this study, map distances were reported in θ distances, where θ is equal to the incidence of recombination between two markers with no correction for the possibility of multiple crossovers. This is because the established linkage groups are likely not yet large enough to justify such a correction. This is, however, something to be considered as more markers are added in future studies.

In the female-specific map, two groups (LG2F and LG5F) were found to contain conflicting marker order between families. This prevented the merging of the two groups into a family-averaged map. Although it is possible that these represent actual chromosomal inversions, it is more likely that the conflicting result is a function of missing or distorted genotypic data associated with these markers. While chromosome inversion polymorphism has been documented in fish species, it is relatively rare due to the small size of the typical fish chromosome (Gold *et al.* 1979). It is expected that these conflicts will be resolved with further mapping experiments.

Differences in rates of recombination between families, hypothesized to be a result of natural variation within and among populations, have been observed in such species as the Pacific oyster (*Crassostrea gigas*) and brown trout (*Salmo trutta*) (Hubert and Hedgecock 2004, Gharbi *et al*. 2006). Significant family-specific differences in recombination between adjacent microsatellites were identified in both same-sex pairs of parents across families, but only the male parents showed a significant value when recombination rates were averaged across all adjacent markers. This result seems to be caused by the presence of a few very large differences, most commonly found where Family B shows zero or a very low level of recombination, while the other family has a much higher incidence of recombination between the same pair. As such, there is not a clear trend of family-specific differences. The statistical significance of the Family A:Family B average across male parents is thus more likely due to the presence of these large outliers than actual differences between families. As more marker pairs are added

and more comparisons become available, a clearer examination of this relationship will be possible.

Sex-specific differences in recombination rates have been reported for a number of fish species, including Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis* spp.), and zebrafish (*Danio rerio*) (Moen *et al*. 2004, Waldbieser *et al*. 2001, Lee *et al*. 2005, Singer *et al*. 2002). There are a variety of hypotheses that attempt to explain this phenomenon, including hypotheses invoking sexual selection and pleiotropy as potential causes, among others (Hedrick 2007). The majority of studies in fish species that have included this type of analysis have often reported higher recombination rates in females, sometimes to a very large extent, as in the case of Atlantic salmon (Moen *et al*. 2004) and rainbow trout (Sakomoto and Danzmann 2000). Also, linkage studies in multiple species, including zebrafish (Singer *et al*. 2002) and humans (Kong *et al*. 2002), have reported that females tend to have higher incidence of recombination near the centromere, while males tend to have higher recombination near telomeric regions. Analysis of available adjacent loci in this study revealed that on average, recombination rates are higher in the male parent for both families. This result was determined to be significant for both families following contingency G-tests of the averaged ratios. The recombination ratio (♀:♂) for Family A was estimated at 0.593, and the same ratio for Family B was estimated at 0.550. This result is supported by many more significant differences between *individual* marker pairs, as was not the case in the cross-family comparisons. However, it is important to note that the low

availability of adjacent markers to compare (due to a relatively small number of markers utilized in the study) may result in data skewed in the direction of more "weighty" outliers.

Utility of a microsatellite-based linkage map

The efficiency of breeding programs is of great interest to stock enhancement and culture operations. In recent years, identification and mapping of quantitative trait loci (QTLs) has become a popular method of increasing the efficiency of traditional artificial selection programs in many agricultural species, most notably in livestock and food crops (Liu 2007). QTL mapping is based on the principal that most traits of interest to breeding programs are controlled by a large number of loci that contribute a small percentage to overall phenotype. Mapping of these QTLs can be accomplished by observing non-random linkage associations with polymorphic genetic markers such as microsatellites (Liu and Cordes 2004). Once these linkage relationships are established, advantageous alleles at various QTLs can be incorporated into a breeding program with more precision (and in less time) than in traditional breeding programs. For hatcheries, which seek to optimize various traits relating to growth rate, susceptibility to cold temperatures, and disease resistance, among others, QTL selection (generally called marker-assisted selection) is much more efficient than traditional selection programs, which often take many generations to generate significant phenotypic improvement (Guimaraes 2007). A first and crucial step in the development of marker-assisted

selection programs is the creation of a dense map of molecular genetic markers such as microsatellites (Karlsson *et al*. 2007).

Moderately-dense linkage maps also have applications in studies of population genetics. Because most microsatellite markers are thought to be neutral with respect to selection, it is possible, after genotyping many individuals at multiple markers, to statistically identify markers that behave differently with respect to patterns of variation within and between populations. These "outlier loci" can then be used to test for the evolutionary phenomena (selection, etc.) that are causing these abnormal patterns of variation (Luikart *et al.* 2003). This is done by identifying or inferring the presence of a gene or gene complex under selection, for instance, that has a tight linkage relationship with the outlier marker. This type of study can potentially provide information that holds a great deal of importance for management and conservation efforts.

Future directions and conclusions

This study represents a step in the generation of a moderately-dense linkage map for red drum. Mapping of additional markers should resolve many of the problems associated with a low-density linkage maps, which include non-resolution of all linkage groups, existence of markers with no linkage relationships, and conflicts in marker ordering caused by a low degree of linkage information. With respect to the analysis of differences in recombination rates, the addition of markers will allow the analysis of

more adjacent, pair-wise combinations. This should result in stronger inferences regarding differential recombination rates in the species.

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